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Competing Isogenic *Campylobacter* Strains Exhibit Variable Population Structures In Vivo[†]

Chris Coward,^{1*} Pauline M. van Diemen,² Andrew J. K. Conlan,³ Julia R. Gog,³ Mark P. Stevens,² Michael A. Jones,⁴ and Duncan J. Maskell¹

Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom¹; Institute for Animal Health, Division of Microbiology, Compton Laboratory, Compton, Newbury, Berkshire, United Kingdom²; Department of Applied Mathematics and Theoretical Physics, University of Cambridge, Cambridge, United Kingdom³; and School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington, Nottingham, United Kingdom⁴

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Consumption of poultry contaminated with *Campylobacter jejuni* is a risk factor for human gastrointestinal disease. The rational development of control strategies for *Campylobacter* within chickens requires an understanding of the colonization process at the molecular and population levels, both within and between hosts. Experiments employing competing strains of *Campylobacter* have been used to investigate colonization. Implicit in these studies is the assumption that the behavior of competing strains is reproducible between experiments. Variability in the recovery of mutants from the chicken gastrointestinal tract during signature-tagged mutagenesis studies demonstrated that this is not always the case. To further investigate this phenomenon in the absence of confounding factors due to phenotypic differences between mutants, we constructed individually identifiable wild-type isogenic tagged strains (WITS) that have indistinguishable phenotypes in pure culture. By using mixtures of WITS, it is possible to monitor the relative amounts of subpopulations of essentially wild-type bacteria. Using a 2-week-old chicken model of colonization, we observed unpredictable variations in population structure both within and between experiments, even in the simplest case of two competing strains. This variation occurred both when birds were simultaneously infected with two WITS and when birds inoculated with different WITS were cohoused. We present evidence for founder effects during initial colonization with subsequent bird-to-bird transmission. We suggest that these and phenotypic variation contribute to the observed variability. These factors render simple models of colonization which do not take them into account inappropriate for *Campylobacter* and impact the planning and interpretation of competition experiments using this organism.

Campylobacter jejuni is a major cause of food-borne gastrointestinal disease in the developed world, with around 45,000 laboratory-confirmed cases per year reported in the United Kingdom alone (12) and an associated economic cost greater than £65 million (14). A major source of human infection is the consumption of contaminated meat, particularly poultry (14). *Campylobacter* is a commensal organism in avian species, with the highest levels of colonization found in the ceca of infected birds (2). Contamination of poultry flocks can occur from multiple sources (1, 14), the relative significance of which is still unclear; however, the population dynamics of *Campylobacter* within and between hosts are still poorly understood (5).

Research into the molecular mechanisms of *C. jejuni* colonization has used a variety of models including oral infection of 1-day-old and 2-week-old chickens (9, 13, 30), ferrets (40), neonatal pigs (25), and mice (38), as well as more artificial systems such as rabbit ileal loops (25, 34). Often, individual mutant *C. jejuni* strains are used in the infections to compare maximal levels of colonization and dose-response relationships to the wild-type parent. Such studies have revealed the impor-

tance in colonization of bacterial factors such as the flagella and capsular polysaccharide (41). A smaller number of publications have used mixed infections, where the model is challenged simultaneously with more than one strain. These studies usually consider the competitive index (CI) of one strain to another, typically enumerating the different bacteria by means of differential antibiotic selection. The precise definition of the CI varies somewhat between studies, but the CI essentially measures the ratio of one strain to another in the model and compares it to that of the inoculum. This has been used to investigate succession of *C. jejuni* and *Campylobacter coli* (6), to examine the effect of bacteriophage on *C. jejuni* strains in chickens (32), and to compare the colonization ability of *C. jejuni* isolates (4, 19, 33), often with particular emphasis on the possibility of competitive exclusion as a control strategy. Comparisons of mutants versus wild type have also been used to demonstrate the importance of particular genes in colonization (25, 34, 38), to establish an advantage for fluoroquinolone-resistant bacteria in colonization even in the absence of antibiotic selection (22), and to validate an insertional gene expression system for *C. jejuni* (18). Signature-tagged mutagenesis (STM), in which pools consisting of a large number of mutants are simultaneously screened through a model, has been successfully applied to a number of species (23) and recently attempted for *C. jejuni* in chickens (9, 13). For *C. jejuni* colonization in 2-week-old chickens (9) and 1-day-old chicks (13), STM is confounded by the high-frequency random loss of colonization-proficient mutants from

* Corresponding author. Mailing address: Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom. Phone: 44 1223 765811. Fax: 44 1223 337610. E-mail: cc122@cam.ac.uk.

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pools and by marked between-bird and between-experiment variations during mixed infections, including instances when birds infected with different mutant strains were cohoused (9). High-frequency loss of mutants was not observed when STM screens were conducted with *Salmonella enterica* serovar Typhimurium using the same model of 2-week-old chicken colonization (24), suggesting that these observations may be a consequence of the nature of *C. jejuni* colonization. We hypothesized that loss of viable bacteria following inoculation (population bottlenecks), transmission of bacteria between birds, and phase variation leading to adaptation for colonization contributed to this variation. All of these factors are likely to be important and could result in unpredictable population structures arising during mixed infections. If this variation is a general feature of competition studies performed using *Campylobacter* spp., caution must be applied in analyzing the data generated from such experiments.

Analysis of previously published work in relation to this experimental variation is confounded by the potential for phenotypic differences between the bacteria used, either because different isolates were used or because a wild type was being compared to mutants. Additionally, the hybridization methods used in STM studies (9, 13) are of low sensitivity, and given the high *Campylobacter* loads present in the ceca, up to $\sim 10^{10}$ CFU/g, it is possible that mutants undetected in these assays were in fact present at relatively high total numbers. In an attempt to remove the potential for phenotypic differences we constructed wild-type isogenic tagged strains (WITS) of *C. jejuni*, which differ from each other only in the sequence of a 40-bp DNA tag inserted into a pseudogene. The relative proportion of these strains in a population can then be determined by quantitative PCR (qPCR) at high sensitivity and specificity, enabling discrimination of WITS over many orders of magnitude, with high sample throughput. As the WITS differ by only the sequence of the DNA tag, we can essentially monitor the structure of competing subpopulations within a single strain. We used a 2-week-old chicken model of colonization to conduct mixed infection experiments and showed extensive between-bird and between-experiment variation, demonstrating that simple colonization models are not appropriate for this organism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* strain M1 was obtained from D. G. Newell (Veterinary Laboratories Agency). This strain is a natural poultry isolate with no reported plasmids; it colonizes chickens to a high level, causes disease in humans, and was previously used by us in STM studies (9). *C. jejuni* was routinely cultured at 42°C on Muller-Hinton (MH) agar supplemented with 5% horse blood (Sigma, Poole, United Kingdom) with 5 µg/ml trimethoprim under standard microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) in a MACS VA500 Variable Atmosphere Work Station (Don Whitley, Shipley, United Kingdom). *Escherichia coli* DH5α was used as the host strain during plasmid construction and was grown at 37°C on Luria-Bertani agar or in Luria-Bertani broth. Where necessary for selection, medium was supplemented with chloramphenicol (10 µg/ml) or ampicillin (100 µg/ml). Long-term storage of bacteria was at -80°C in Microbank vials (Prolab Diagnostics, Neston, United Kingdom).

Selection of chromosomal location for insertion of DNA tags. WITS were generated by insertion of 40-bp DNA tags into a pseudogene in *C. jejuni* strain M1. The genome sequence of this strain was not determined when this work was conducted; putative pseudogenes in M1 were identified based on the published NCTC11168 sequence (11, 27). To minimize the possibility of polar effects on

downstream genes caused by insertion of the tag, only pseudogenes that were not directly upstream of open reading frames were considered. Six of the 19 pseudogenes in the NCTC11168 sequence (11, 27) matched this criterion: cj0046, cj0501, cj0742, cj0752, cj1470c, and cj1528. We rejected cj0501 and cj1470c as they both possessed a potential coding sequence at their 5' ends. cj0742 was rejected as sequence from a bacterial artificial chromosome clone showed that the multiple stop codons and frameshifts present in the NCTC11168 gene are not present in M1 (E. Kay, personal communication). cj0752 was rejected as it was not detected in the same chromosomal location as in NCTC11168 (E. Kay, personal communication), and no product could be amplified by PCR from M1 chromosomal DNA using primers designed to this locus based on the NCTC11168 sequence (data not shown). cj1528 was also rejected as PCR amplification and partial sequencing showed that the multiple stop codons present in the 5' part of the NCTC11168 gene were not present in M1 (data not shown). PCR amplification and sequencing of a 1,541-bp region of the M1 chromosome containing cj0046 (GenBank accession no. EU008092; see below) showed that this locus was more similar to the *C. jejuni* RM1221 sequence (7), with 99% identity over the available sequence, than that of NCTC11168. Assuming that the M1 cj0046 start codon is the same as that predicted for the RM1221 gene, i.e., 5'-ATG-3' at position 70 in GenBank EU008092, the M1 gene has 15 stop codons over its length; hence, this locus is highly likely to be a pseudogene in this strain. An MfeI restriction site at nucleotide position 287 was suitable for insertion of the sequence tag cassettes. The M1 genome has subsequently been partially sequenced and is available as a collection of contigs (Wellcome Trust Sanger Institute, http://www.sanger.ac.uk/Projects/C_jejuni). The cj0046 region sequence obtained in the present study and that obtained in the partial genome sequence were identical.

Design and construction of DNA tags. Plasmids used and constructed in this study are listed in Table S1 in the supplemental material. To enable construction of WITS by the insertion of tags into the chromosome and subsequent discrimination by qPCR, tag cassettes (see Fig. S1 in the supplemental material) were generated as follows: (i) EcoRI sites were used to generate MfeI-compatible ends for cloning into the cj0046 locus; (ii) chloramphenicol acetyltransferase (*cat*) was used for an antibiotic resistance cassette; and (iii) DNA tags were inserted 5' to the *cat* gene to create priming sites for qPCR using Sybr green fluorescent dye incorporation (see Fig. S1 in the supplemental material). Tags were kindly supplied by D. Holden (Imperial College, London, United Kingdom) and had previously been sequenced by us (9). We screened the tag and *cat* sequences for suitable qPCR priming sites using the parameters of Inglis et al.; ideally, an amplicon of 75 to 150 bp; primers 18 to 25 nucleotides in length with a 50 to 60% GC content; a thermal denaturation midpoint temperature of 58 to 60°C; and no more than two G or C residues in the last five 3' nucleotides (15). Two tags were selected that yielded priming sites that matched these parameters, with the exception of the amplicon length, which was 180 bp. Standard methods were used for molecular cloning (31). Primer sequences for the PCRs are shown in Table S2 in the supplemental material. DNA sequences were confirmed at each step, with sequencing being conducted by GeneService (Cambridge, United Kingdom). Preparation and purification of DNA were performed by using Qiagen kits (Qiagen, Crawley, United Kingdom). pUC19-derived plasmids pCC012 (tag 1) and pCC019 (tag 2) containing tagged *cat* cassettes were generated by PCR amplification of the chloramphenicol resistance cassette from pRY111 (39) using primers CC059 with either CC060 (tag 1) or CC063 (tag 2), followed by digestion with EcoRI and cloning into the similarly digested plasmid pUC19. PCRs were performed using the FailSafe system (Epicenter Biotechnologies, Madison, WI) with buffer B at an annealing temperature of 55°C and with an extension time of 1.5 min. To verify that these tags were suitable for qPCR prior to construction of WITS, pCC012 and pCC019 plasmid DNA was purified and used as a template in standard PCR and qPCR (not shown).

Generation of WITS. WITS CC001a (tag 1), CC001b (tag 1), and CC003a (tag 2) were generated by moving the tagged *cat* cassettes into *C. jejuni* M1 cj0046 using suicide delivery plasmids pCC027 (tag 1) and pCC031 (tag 2) containing the tagged *cat* cassettes inserted into the MfeI site of cloned M1 cj0046 (see Fig. S1 in the supplemental material). Firstly, pCC024 was generated by cloning the *C. jejuni* M1 cj0046 gene and 5' flanking region as a BamHI fragment generated by PCR from purified M1 chromosomal DNA amplified using primers CC076 and CC077 at an annealing temperature of 53°C using *Pwo* polymerase (Roche Diagnostics GmbH, Mannheim, Germany) into similarly digested pUC19. Suicide delivery plasmids pCC027 (tag 1) and pCC031 (tag 2) were generated by cloning tagged *cat* cassettes excised from pCC012 or pCC019 with EcoRI into MfeI-digested pCC024. Tagged *cat* cassettes were moved onto the *C. jejuni* M1 chromosome via double homologous recombination following natural transformation of M1 using pCC027 or pCC031 by a plate biphasic method adapted from van Vliet et al. (36). Correct insertion of the tagged *cat* cassettes was confirmed

by PCR and Southern blotting (data not shown). Pulsed-field gel electrophoresis of chromosomal DNA digested with *Sac*II, *Sal*I, or *Sma*I showed that no large-scale genomic alterations had occurred (data not shown). CC001a and CC001b were independently derived isolates containing tag 1 generated on separate occasions. To verify that the WITS behaved indistinguishably in vitro, growth rates and final overnight bacterial densities were determined following incubation with shaking of a $1:10^6$ dilution of an overnight culture in brain heart infusion broth, and motility was assessed by measuring the diameter of colonies following inoculation into 0.4% agar MH plates.

qPCR for determining proportions of WITS. Total DNA was prepared from *Campylobacter* harvested either from 48-h plate cultures or from pellets from liquid culture using a DNeasy blood and tissue kit (Qiagen). DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and $\sim 10^6$ genome copies were used in qPCR. qPCR was performed in a Rotor-Gene 2000 (Corbett Life Science, Sydney, Australia) using QuantiTect Sybr green (Qiagen) dye incorporation. Primers were CC069 and either CC065 (tag 1) or CC067 (tag 2). Reaction mixtures had a total volume of 25 μ l with 5 μ l of template DNA and 1 μ mol of each primer; cycling conditions were 95°C for 15 min and 30 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 20 s, with a final melt curve analysis step from 65°C to 95°C. All samples were run in duplicate, and standard curves for CC001 and CC003 total DNA were included in duplicate in each run. Data were analyzed using Rotor-Gene software, version 6.0, with dynamic tube normalization. A linear relationship between threshold cycle number and \log_{10} genome copies over a range of ~ 10 to 10^7 copies was obtained (data not shown). Linear regression generated standard curves for \log_{10} genome copies as a function of threshold cycle number. For each sample, the regression lines were used to generate predictions of either the ratio of genome copies of CC001 to CC003 as \log_{10} (genome copies of CC001/genome copies of CC003) or the CI of CC001 with respect to CC003 as the \log_{10} CI, calculated as the \log_{10} (genome copies of CC001_{sample}/genome copies of CC003_{sample})/(genome copies of CC001_{inoculum}/genome copies of CC003_{inoculum}). Measurement errors for these values were calculated as 95% confidence intervals using variances derived from the standard curves. Data are plotted graphically (see Fig. 1 to 3; see also Fig. S3 and S4 in the supplemental material); numerical values for ratios, CIs, and confidence intervals can be found in the supplemental material (see Tables S4 to S9 in the supplemental material).

Experimental animals and challenge model. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the local Ethical Review Committee.

Specific-pathogen-free Light Sussex chickens were produced at the Institute for Animal Health, Compton, United Kingdom. To standardize the initial gut flora, chicks were inoculated orally on the day of hatch with 0.1 ml of a *Campylobacter*-free adult gut flora preparation as described previously (16). Birds were housed in groups of three or five in cages in high-biosecurity accommodation and fed a vegetable-based diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom) ad libitum.

Two-week-old birds with a developed gut flora were inoculated orally with 0.3 ml of an MH broth culture containing the *Campylobacter* WITS at doses and ratios as required. Within an experiment, all groups were in spatially separated cages, independent of each other. For the transmission experiments, two birds in each group of five were inoculated with a single WITS ($\sim 10^7$ CFU) while the others were uninoculated. In all other experiments, all the birds within an experiment received the same inoculum consisting of a mix of WITS in various proportions. The actual ratio of WITS in the inoculum was determined by qPCR. All birds within a cage were sacrificed on the day appropriate for the experiment, and the numbers of viable *Campylobacter* bacteria per gram of cecal and ileal contents were enumerated postmortem by suspending the intestinal contents in $1\times$ phosphate-buffered saline (1 ml/g of contents) and plating 10-fold serial dilutions onto *Campylobacter* charcoal differential agar medium with chloramphenicol. The sensitivity of detection was taken to be 10^2 CFU/g. For determination of the proportion of WITS, bacteria were harvested by scraping into $1\times$ phosphate-buffered saline from five plates inoculated with 100 μ l of the 1/10 dilution and then stored at -70°C prior to DNA extraction and qPCR. In some cases only one WITS was detected; here, the limit of detection was determined by the cecal load: for loads $>10^7$ CFU/g the sensitivity of the qPCR was the limiting factor (1 in $\sim 10^6$); for loads $<10^7$ CFU/g the limiting factor was the number of colonies analyzed (equal to cecal load/20). Cloacal swabbing was attempted to generate longitudinal sampling data, but the level of *Campylobacter* bacteria recovered at each time point was too varied and low to enable an accurate determination of the relative amounts of each WITS present in the birds. Similarly, ileal sampling to examine variation along the gastrointestinal tract did not reproducibly yield enough *Campylobacter* bacteria for accurate assessment.

TABLE 1. WITS and wild-type M1 parent strain demonstrate indistinguishable phenotypes in terms of growth, motility, and in vivo dose response^a

Strain	Growth rate (no. of divisions/h)	Overnight density (\log_{10} CFU/ml)	Motility (mm)	Dose-response data	
				Dose (\log_{10} CFU/bird)	Cecal load (\log_{10} CFU/g)
M1	1.15 ± 0.08	ND	23 ± 2	ND	ND
CC001a	1.22 ± 0.06	9.0 ± 0.01	23 ± 1	3.8	8.7 ± 0.6
				5.9	8.5 ± 1.0
				8.0	8.7 ± 0.8
CC001b	1.17 ± 0.07	8.9 ± 0.16	24 ± 2	3.0	2.0 ± 3.3
				4.8	8.2 ± 0.4
				6.6	8.5 ± 0.02
CC003a	1.19 ± 0.14	9.1 ± 0.06	22 ± 2	2.8	NDC
				4.2	7.8 ± 0.4
				6.3	8.3 ± 0.8

^a Errors are 95% confidence intervals. ND, not done; NDC, no detectable colonization.

Nucleotide sequence accession number. The nucleotide sequence of the *C. jejuni* M1 cj0046 locus has been deposited in the GenBank database under accession number EU008092.

RESULTS

Generation of individually identifiable, phenotypically indistinguishable strains and validation of their discrimination by qPCR. Individually identifiable WITS were generated by inserting distinct 40-bp DNA signature tags and a selectable marker into a pseudogene of *C. jejuni* strain M1. WITS CC001a and CC001b were independently derived isolates harboring tag 1; WITS CC003a harbored tag 2. The WITS could be discriminated by tag-specific PCR. In vitro growth curves and motility assays showed no differences between the wild-type and WITS (Table 1). The M1 parent colonizes chickens to a high level, i.e., $>1 \times 10^8$ CFU/g of cecal contents following oral inoculation with $\sim 1 \times 10^7$ CFU (9). Dose-response experiments showed all WITS colonized to $\sim 1 \times 10^8$ CFU/g of cecal contents at day 7 postinfection at inoculum doses greater than $\sim 1 \times 10^4$ CFU (Table 1). To verify that the relative numbers of differently tagged bacteria could be determined by qPCR, cultures of differently tagged WITS were mixed in various ratios, chromosomal DNA was prepared, and the ratios as determined by qPCR and colony count were compared (see Fig. S2 in the supplemental material). As expected, we obtained a linear relationship between the \log_{10} (qPCR ratio) and the \log_{10} (count ratio), indistinguishable from the identity relationship; i.e., the ratios derived from qPCR were indistinguishable from the ratios derived by colony count. To verify that the WITS were equally competitive in vitro, competition experiments between CC001a or CC001b and CC003a were performed in liquid culture. qPCR was used to assess the amount of each WITS present in a 1:1 (vol/vol) mix and after 24 h of growth of a $1/10^6$ dilution of the mix, the logarithm of CI, \log_{10} CI, was calculated. A \log_{10} CI of 0 would show that both WITS were equally competitive. For CC001a versus CC003a we obtained a \log_{10} CI of -0.20 ± 0.32 (95% confidence interval); for CC001b versus CC003a we obtained a \log_{10} CI of 0.14 ± 0.32 ; i.e., we could not detect a difference in the ability of the WITS to compete in vitro.

Having verified that the WITS behaved indistinguishably *in vitro*, we next used mixtures of them to investigate the population dynamics occurring within subpopulations following oral inoculation of 2-week-old chickens harboring standardized gut flora. For these experiments the relative amounts of WITS in the ceca of infected birds was determined by performing WITS-specific qPCR on WITS DNA prepared from bacteria harvested from plates inoculated with 1/10 dilutions of total cecal contents. To avoid the requirement for this *in vitro* growth step, attempts were made to perform WITS-specific qPCR on total DNA directly extracted from the cecal contents, but this was unsuccessful due to the very low level of *Campylobacter*-specific DNA present as a proportion of the total DNA extracted.

We present the data in two main sections, first to assess transmission of WITS between individually inoculated cohoused birds and then to monitor population structures in birds coinoculated with both WITS. For an overview of all the following *in vivo* experiments, see Table S3 in the supplemental material.

Transmission of *Campylobacter* between individually inoculated, cohoused birds can lead to cocolonization and replacement. During previous colonization experiments using STM, we observed that when birds inoculated with single, distinct mutants were cohoused, there was between-experiment variation in the mutants recovered (9). Some birds were colonized with the mutant with which they were inoculated, some harbored a different mutant, and some were colonized by more than one strain. We asked whether this phenomenon was also observed when there was no defined genetic difference between the colonizing bacteria.

We conducted two independent experiments (I-1 and I-2) to simultaneously monitor the ability of WITS CC001a and CC003a to compete when coinoculated into chickens and to transmit between individually inoculated cohoused birds. To assess competition following coinoculation, two groups of five birds (one group for each sampling at day 3 and day 7) were inoculated with a 1:1 (vol/vol) mix of the WITS at a total dose of $\sim 10^7$ CFU, and the relative numbers of WITS were determined postmortem by qPCR (Fig. 1 shows the data graphically). The width of the data points corresponds to 95% confidence intervals. For full numerical data, see Tables S4 and S5 in the supplemental material). It appeared that CC003a was more competitive than CC001a. For experiment I-1, in all the birds of the day 3 group, we could detect only CC003a, and in all the birds of the day 7 group there was at least a 10^4 -fold overabundance of CC003a. For experiment I-2, the results were different; at day 3 all birds harbored both WITS but their relative amounts varied between birds over 4 orders of magnitude. For the day 7 group, all birds harbored only detectable CC003a. Clearly, even if CC003a had a competitive advantage compared to CC001a, the magnitude of this advantage varied both between birds within a group and between experiments.

To simultaneously monitor transmission between birds inoculated with a single WITS, four groups of five birds (two for each sampling at day 3 and day 7) were used. In each of these groups, one bird received CC001a alone, one received CC003a alone, and three were uninoculated. The relative numbers of WITS were determined by qPCR postmortem (Fig. 1; see also Tables S4 and S5 in the supplemental material). It was clearly

seen that both WITS were able to colonize an inoculated bird and to transmit both to uninoculated animals and to birds that had been inoculated with the reciprocal WITS. The distribution of WITS within birds at the same time point varied between groups that received the same inocula and did not necessarily reflect the ability of the WITS to compete when coadministered, as demonstrated by experiment I-1. Here, all the birds in the mixed inoculum competition experiment had at least a 10^4 -fold overabundance of CC003a. At day 3 in the transmission experiment for cage 1, in bird i (inoculated with CC001a alone) we detected only CC001a, as we also did for the uninoculated bird iii. The other three birds including bird ii (inoculated with CC003a alone) had both WITS in various proportions. For cage 2, all birds harbored both WITS, although only the bird inoculated with CC001a alone had an overabundance of CC001a. At day 7 all but two birds harbored both WITS, and again the relative amounts of the WITS varied over many orders of magnitude. In experiment I-2, it was striking that all of the birds in both of the day 7 groups harbored only detectable CC003a, including even those that were originally inoculated with CC001a, which clearly demonstrates that complete replacement of one WITS by the other is possible (at least to the limit of detection in the assay, 1 in $\sim 10^6$). To determine whether the CC003a strain in this experiment had acquired an obvious *in vivo* competitive advantage by virtue of an increase in motility, we conducted motility assays on CC003a bacteria recovered from the ceca of birds in one of the day 7 groups (zone of motility of 18 ± 3 mm). No difference was detected between these and the strains used to inoculate (CC001a, 19 ± 3 mm; CC003a, 20 ± 2 mm).

To elucidate further the timing of the transmission events and to examine between-experiment variation, two additional experiments (I-3 and I-4) were performed using groups of five birds (see Fig. S3 and Table S6 in the supplemental material) with sampling at days 1, 3, and 7 postinoculation. In both experiments, there was no detectable transmission of WITS to either the inoculated or uninoculated birds at day 1. At day 3, five out of eight inoculated birds harbored only the WITS used to infect, while the uninoculated birds had both WITS in variable proportions or CC003a alone. At day 7, half (4/8) of the inoculated birds had only the WITS used to inoculate, while the remaining inoculated birds had both strains in various proportions. The uninoculated animals harbored both WITS in various proportions, CC001a alone (two birds), or CC003a alone (one bird).

Taken together, these experiments demonstrate that transmission of WITS between cohoused birds could not be detected until after at least 24 h. They also show that birds colonized by one WITS can subsequently acquire a second WITS and that complete replacement of one WITS by another is possible. Finally, the variation in population structure between birds within a group, between groups inoculated with the same bacteria, and between experiments shows that any possible phenotypic difference between WITS as evidenced by the mixed inoculum competition experiments cannot be solely responsible for the final distribution of WITS. Stochastic effects during transmission are likely to play a significant role. These include the possibility of bottlenecks (population crashes) during the colonization process leading to founder effects, *in vivo* adaptation/selection, and possible phenotypic variation during

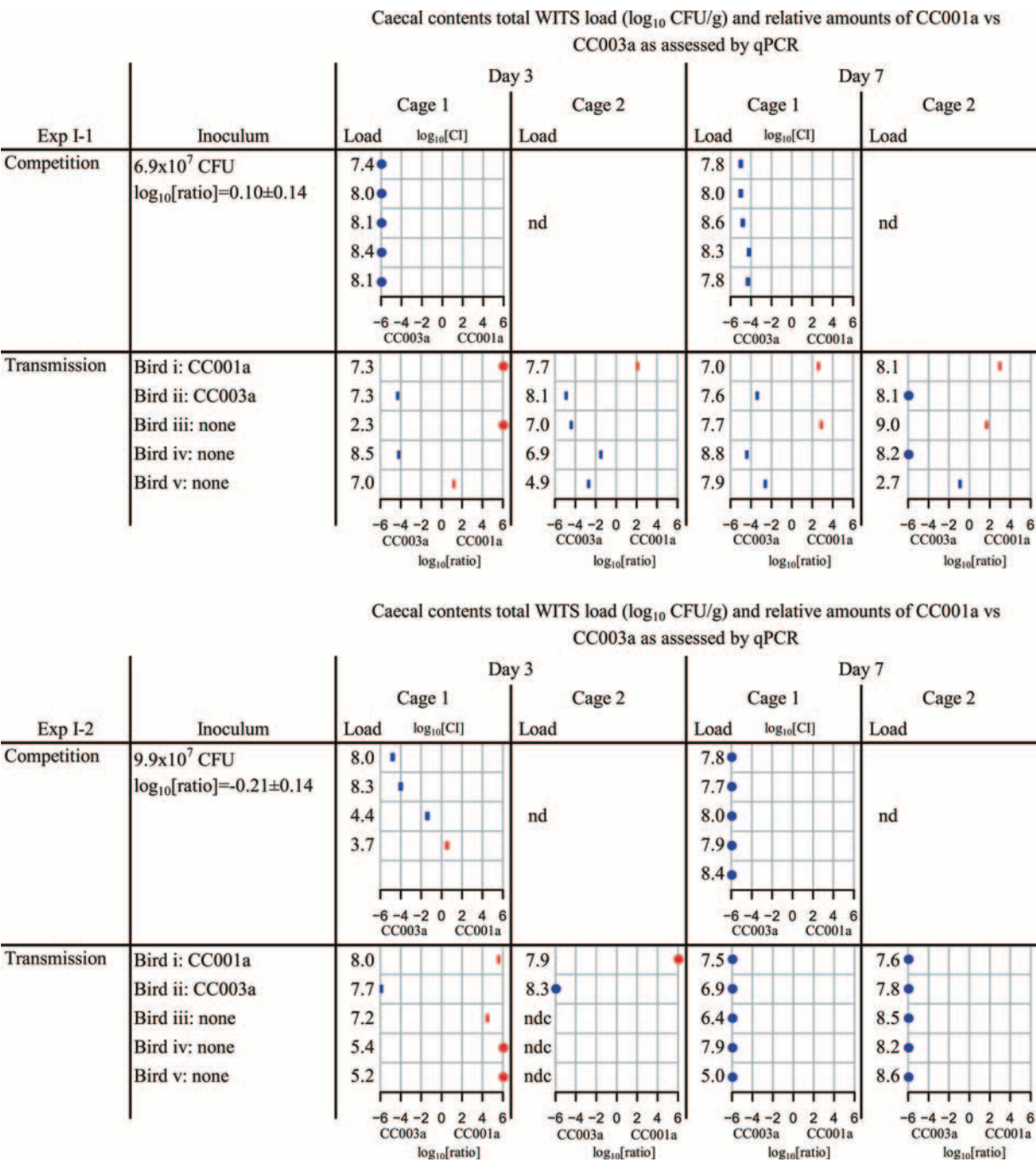


FIG. 1. Population structure of WITS during competition and transmission experiments. Data represent the total cecal loads of WITS and relative amounts of WITS CC001a and CC003a postmortem in the ceca of birds at the indicated days postinfection. The predominating strains are shown on the axes. Red rectangles, bias in favor of CC001a (log₁₀ CI of >0); blue rectangles, bias in favor of CC003a (log₁₀ CI of <0). The width of the rectangles corresponds to 95% confidence intervals. Where only one strain was detected, this is denoted by a filled circle. nd, not done; nde, no detectable colonization; vs, versus.

culture or infection. To begin to investigate some of these possibilities we conducted a more extensive series of mixed inoculum competition experiments.

The proportion of WITS present in chicken ceca following oral infection with a mixed population is not predictable from the inoculum. In a simple colonization model (which ignores bottlenecks during the colonization process, in vivo adaptation/

selection, and phenotypic variation during inoculum preparation), the ratio of two coadministered phenotypically indistinguishable strains at all time points should be the same as that in the inoculum. The mixed infection competition experiments in I-1 and I-2 suggested that CC001a might be somewhat less competitive than CC003a in vivo. The following experiments used an independently derived isolate of this strain, CC001b,

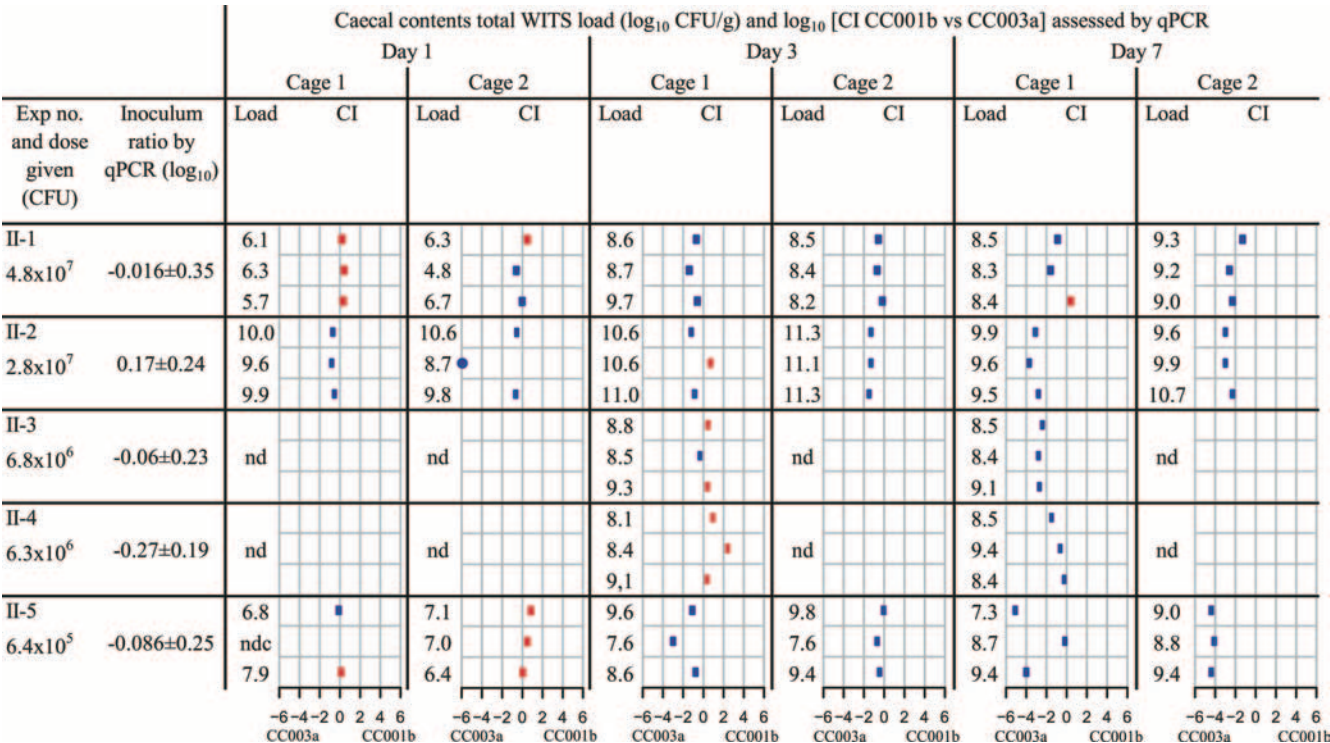


FIG. 2. Population structure of WITS in the ceca of birds following inoculation with a 1:1 mix of WITS at a range of total doses. Data represent the caecal loads of WITS and CI of CC001b versus CC003a in individual birds at days 1, 3, and 7 postinoculation with a 1:1 (vol/vol) mix of the two strains at the indicated doses. The predominating strains are shown on the axes. Red rectangles, bias in favor of CC001b (log₁₀ CI of >0); blue rectangles, bias in favor of CC003a (log₁₀ CI of <0). The width of the rectangles corresponds to 95% confidence intervals. Where only one strain was detected, this is denoted by a filled circle. nd: not done; ndc: no detectable colonization; vs, versus.

which was more able to compete in vivo with CC003a than was CC001a as demonstrated below. Experiments were conducted (II-1 to II-5 and III-1 to III-3) in which 2-week-old chickens were orally infected in groups of three with 1:1 (vol/vol) mixes of *C. jejuni* WITS CC001b and CC003a at doses ranging from ~10⁴ to ~10⁷ CFU, and the amounts of each WITS present in the ceca at days 1, 3, and 7 postinfection were determined by qPCR (Fig. 2 and 3; see also Tables S7 and S8 in the supplemental material). Inocula for all experiments were prepared from independent cultures of WITS, with the exception of III-1 and III-2.

For inoculum doses greater than ~5 × 10⁵ CFU (experiments II-1 to II-5) (Fig. 2; see also Table S7 in the supplemental material), all birds were colonized by both WITS at all time points, with the exception of one bird at day 1 in experiment II-2, in which only CC003a was detected, and one bird at day 1 in experiment II-5, which had no detectable colonization. This reassured us that there was no large difference in competitiveness between CC001b and CC003a, whereas we could not be sure of this for CC001a as demonstrated by the data in experiments I-1 and I-2. In general, at day 1 the ratio of the two WITS was similar to that in the inoculum (log₁₀ CI of ~0). At day 7 a bias in favor of CC003a was observed in most experiments (log₁₀ CI of <-1), although this was not evident in all birds. This bias may indicate that CC003a was in fact somewhat more fit for colonization than CC001b despite their indistinguishable in vitro phenotypes. Notwithstanding this possibility, there was extensive variation in the relative amounts of each

WITS between birds within groups, between groups that received the same inoculum, and between experiments using similar inoculum doses.

Striking examples of within-group variation included experiment II-5 (on day 7 for cage 1) where in two birds CC003a predominated by at least 10⁴-fold whereas in one bird the WITS appeared approximately equally competitive. In both experiment II-1 (day 7 for cage 1) and experiment II-2 (day 3 for cage 1), one bird in each group had a slight overabundance of CC001b while in the other birds CC003a predominated. In experiment II-4 (day 3 for cage 1) all birds had a bias in favor of CC001b, but the magnitude of this bias varied over a ~100-fold range.

Variation between groups within the same experiment was clear in experiment II-1: on day 7 for cage 1, one bird had a slight (approximately threefold) overabundance of CC001b, and the other two birds had an overabundance of CC003a (~8-fold and ~40-fold); for cage 2, all birds had a 20- to 400-fold overabundance of CC003a. The variation occurred even though all of these birds received the same inoculum.

Variation between experiments using approximately equal inoculum doses was striking for experiment II-2 (day 7), where CC003a was ~1,000-fold more competitive than CC001b; however, in experiment II-1 (on day 7 for cage 1), this advantage was at best 50-fold. Similarly, experiment II-3 (on day 3 for cage 1) indicated that both WITS were approximately equally competitive while in experiment II-4 (day 3 for cage 1), CC001b had an advantage of up to ~250-fold.

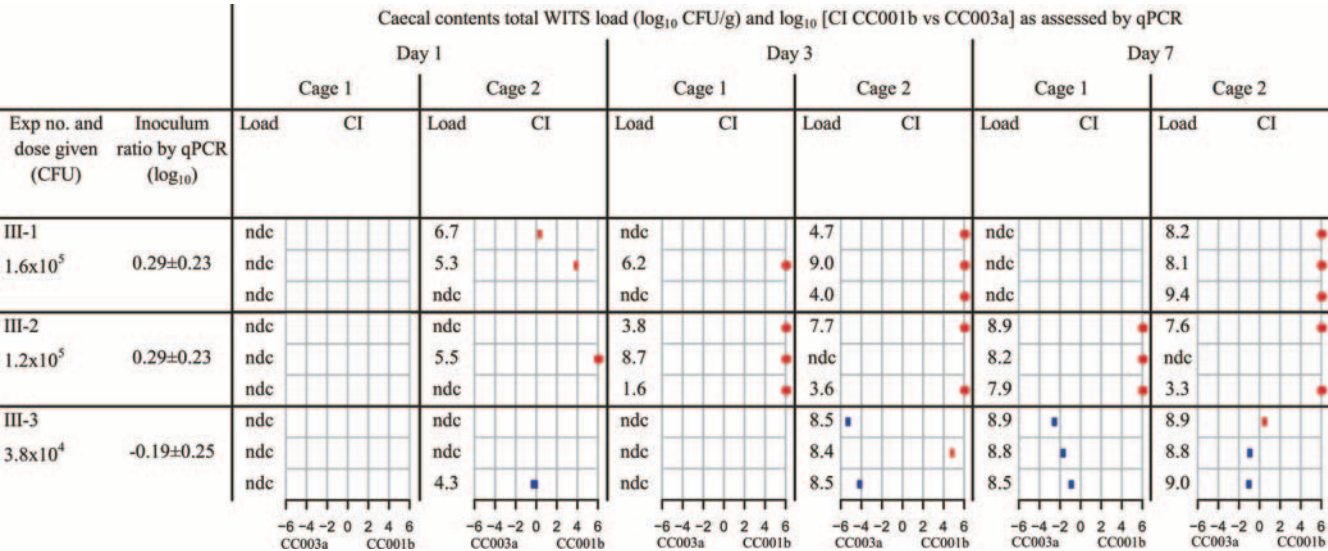


FIG. 3. Population structure of WITS in the ceca of birds following inoculation with a 1:1 mix of WITS at a range of total doses. Data represent the caecal loads of WITS and CI of CC001b versus CC003a in individual birds at days 1, 3, and 7 postinoculation with a 1:1 (vol/vol) mix of the two strains at the indicated doses. The predominating strains are shown on the axes. Red rectangles, bias in favor of CC001b (log₁₀ CI of >0); blue rectangles, bias in favor of CC003a (log₁₀ CI of <0). The width of the rectangles corresponds to 95% confidence intervals. Where only one strain was detected, this is denoted by a filled circle. ndc: no detectable colonization; vs, versus.

Colonization by a single strain following inoculation with a low total dose of WITS suggests a founder effect. The presence of both WITS in all but one of the birds at day 1 following inoculation with >5 × 10⁵ CFU, i.e., before transmission could play a role, demonstrated that viable cells of both strains must have been responsible for initiating colonization. At inoculum doses below 5 × 10⁵ CFU (experiments III-1 to III-3) (Fig. 3; see also Table S8 in the supplemental material), the data were qualitatively different.

First, not all of the birds were colonized, particularly at the early time points. Second, in experiments III-1 and III-2, only CC001b could be detected in all but two of the colonized birds. This clearly demonstrated that CC001b was able to compete effectively with CC003a for colonization and suggests a founder effect—loss of genetic variability (in this cases detected by absence of CC003a) in the colonizing population within an inoculated bird because colonization is initiated by only a few cells. The failure to detect CC003a in these experiments was striking. Transmission of WITS between birds would mean that if any bird within a group had been successfully colonized by CC003a, we would expect this bacteria to be present in most birds by day 7. The approximately twofold bias in favor of CC001b in the inoculum could explain the failure to detect any CC003a as it may have had the result that only viable CC001b bacteria survived to establish colonization, whereas in experiment III-3 the amounts of each WITS in the inoculum of the lower-dose experiment III-3 were more equal.

Inoculation with cultures biased in favor of one WITS resulted in colonization by only one strain even at a high total dose. To further address the possibility that founder effects become important at a dose of around 10⁵ CFU, we inoculated birds with CC001b and CC003a at the ratio 1:100 (vol/vol) at a total dose of ~10⁷ CFU (see Fig. S4 and Table S9 in the supplemental material). Based on the previous results, we pre-

dicted that the overrepresented WITS would always be detected, whereas the minority WITS, present at ~10⁵ CFU, would be absent from some birds. Consistent with this prediction, in the experiments in which CC003a was overrepresented in the inoculum, 45/69 birds were colonized by CC003a alone. This contrasted with the experiments in which >10⁶ CFU of both WITS were inoculated in a 1:1 ratio, and only 1/48 were colonized by this WITS alone. Similarly, where CC001b was overrepresented in the inoculum, 10/69 birds were colonized by CC001b alone, whereas this was never observed in the 1:1 high-dose experiments (i.e., 0/68 birds). The overrepresented WITS was never absent from a colonized bird. Where both WITS were present, CC003a had a competitive advantage (log₁₀ CI of <0) in 63/82 individuals while CC001b had an advantage in only 5/82, again suggesting that this WITS may in fact be somewhat less fit for colonization than CC003a. Nevertheless, in 15/82 animals the two WITS were equally competitive. Because transmission of WITS between birds could obscure instances of initial colonization by only one WITS, we also considered the groups as individual units (i.e., we looked for cases where only one WITS was detected in all birds within a group). When CC003a was overrepresented in the inoculum, 10/24 groups contained CC003a alone; for CC001b the figure was 1/24 groups. When the inoculum ratio was 1:1 at a high dose, the presence of a single WITS within a group was never observed (i.e., 0/22 groups).

DISCUSSION

We have developed a qPCR-based method for monitoring the population structure in competition experiments between strains of *Campylobacter* designed to be phenotypically identical, WITS. This technique facilitates sensitive, high-throughput analysis of the proportion of strains within a sample without

the possibility of phenotypic differences arising from utilizing differential antibiotic selection and enables us to monitor the proportions of subpopulations within a wild-type population.

We used this system to analyze the population structure of *Campylobacter* during colonization of 2-week-old chickens harboring standardized gut flora.

Colonization was initiated by the administration of inocula consisting of known proportions of the two WITS. Establishment of the initial colonizing population of *Campylobacter* in the ceca occurs within 24 h (before bird-to-bird transmission can occur) and results from the first passage of this inoculating population through the gastrointestinal (GI) tract. During transit through the hostile environment of the GI tract, it is likely that there will be a substantial loss of viable bacteria, i.e., a population bottleneck. At low-inoculum doses this could result in a founder effect in the initial colonizing population due to stochastic loss of one or the other WITS from the inoculating population. The colonizing population at later time points will arise both from the initial colonizing population and subsequent acquisition of *Campylobacter* from the cage environment (bird-to-bird transmission). At post-mortem examination we observe a snapshot of these populations in individual birds.

We make two main observations: (i) for two strains with no defined genetic difference (except for the sequence of a 40-bp tag) which are phenotypically indistinguishable in pure culture, it is possible that the strains have different abilities to compete in vivo; (ii) there is extensive variation both within and between in vivo experiments where the two strains are in competition, which has major implications for the way competition experiments are conducted and interpreted.

For the first point, we showed that the WITS we generated had indistinguishable phenotypes in vitro and in vivo when assessed individually. Despite this, in the simplest competition experiments where chickens were inoculated with approximately equal numbers of both WITS at a high total dose ($>5 \times 10^5$ CFU/bird), one (CC003a) in general had a competitive advantage by day 7 postinoculation, although this was not manifested in all birds. This was true even when this WITS was competed with two independently derived isolates of the other WITS (CC001b or CC001a). There is a formal possibility that this apparent advantage of CC003a was due to a phenotypic difference arising due to the difference in sequence of the 40-bp tag inserted during construction. Given that the tag was inserted into an isolated pseudogene, we consider this extremely unlikely. More probable is that any difference in competitiveness may have arisen as a consequence of phenotypic variation of *Campylobacter* during construction of the WITS. *Campylobacter* spp. possess homonucleotide tracts with the potential for length variation due to slipped-strand mispairing during DNA replication (20), leading to changes in gene expression. These variable tracts are predominately located in regions of the *Campylobacter* chromosome encoding factors known to be important in colonization, such as the capsular polysaccharide, lipooligosaccharide, and flagella (8, 10, 11, 17, 21, 26, 27, 28, 35). The frequency of this phase variation has not been extensively studied, although for some loci it is high enough for multiple variants to be present within a single colony (8, 10, 37). During construction of the WITS, it was necessary to pick a single colony in order to verify integration of the tag into the correct chromosomal location. It is possible

that the selected colonies had arisen from or contained bacteria with phenotypic differences that only became apparent when the WITS were competed in vivo. Phenotypic variation could also occur during the experiments themselves, both in vitro during inoculum preparation and in vivo. Alterations in surface-exposed structures could result in differences in interactions with the host immune system, which may lead to unpredictable changes in population structure, particularly if there is a density-dependent component, as recently suggested (33). This could account for some of the variation observed in the colonization experiments.

Concerning the second main point, the variation both within and between experiments, we undertook this study to further investigate variability observed during STM experiments. Briefly, there was between-experiment and between-bird variation in mutants recovered in coinfection experiments both when chickens were inoculated with pools of mutants and when birds infected with single mutants were cohoused (9, 13). Hendrixson et al. also observed experimental variability when birds were coinfecting with a wild-type strain and a streptomycin-resistant derivative (13). Both studies also showed that mutants capable of colonizing when inoculated into birds individually were lost from mixed infections in an unpredictable manner.

Using the WITS we observed extensive variability in both transmission and coinfection experiments that was both qualitative (i.e., whether CC001b or CC003a predominated) and quantitative (the magnitude of bias in favor of a particular WITS) and occurred whenever there was a mixed population of *Campylobacter* within a cage even in the absence of a defined genetic or phenotypic difference between strains. The variability was apparent both within and between groups at high- and low-inoculum doses and where both WITS were present in equal amounts in the inoculum or where there was a 100-fold bias in favor of one WITS. This was true for the simplest case of two competing strains and is likely to be even more apparent when larger numbers are competing, which could account for the inconsistent results obtained during STM studies (9, 13). We hypothesize that bird-to-bird transmission, population bottlenecks, and phenotypic variation contribute to this variability.

We have clearly shown that transmission of WITS between cohoused birds can be detected by day 3 postinfection and can lead to cocolonization in both uninoculated animals and those initially infected with a large dose ($\sim 10^7$ CFU) of a single WITS. There were also examples of complete replacement of one WITS by the other. The observations of cocolonization and replacement in birds inoculated with a single strain show that the final colonizing population is a product both of the initial colonizing population (derived from the inoculum) and transmission from other birds in the cage. Moreover, the ability of a WITS to establish colonization, transmit to other birds in a cage, and compete with the other WITS within the cage did not reflect the ability of the WITS to compete when coinoculated. This was demonstrated in experiment I-1; when CC001a and CC003a were coinoculated, CC003a predominated in all birds, whereas in the transmission groups using the same incubating cultures, the majority of birds harbored both WITS. It is tempting to speculate that in vivo selection for more competitive variants of CC001a had occurred within the birds that were inoculated with this WITS. Upon transmission, these bacteria could more effectively compete with the CC003a bac-

teria present within the group. Adaptation for in vivo growth has previously been observed, with campylobacters recovered from passage through chickens being more able to colonize than the original isolate (3, 16, 29). The transmission process is likely to be subject to various stochastic effects in addition to the potential for adaptation. These include physiological and behavioral differences between chickens, affecting the number of bacteria excreted and ingested (e.g., the amount of shedding, pecking order, and coprophagy), as well as the timing of transmission events. Transmission will also be occurring in the coinfection experiments and is another potential source of variation in the results.

We also present evidence, both from the low-dose 1:1 mixed inoculum experiments and those in which one WITS was over-represented by ~ 100 -fold in the inoculum, that founder effects become important at doses below $\sim 5 \times 10^5$ CFU. When both WITS were present in the inoculum above this level, the initial colonizing populations contained both strains, a result inconsistent with the idea that colonization proceeds from a single ancestor cell following high-dose inoculation. The presence of founder effects at low inoculum doses and when the inoculum was biased in favor of one strain demonstrates that not all of the inoculating population contributes to the initial colonizing population. This is presumably a consequence of a population bottleneck between inoculation and the onset of cecal colonization. This inoculum's cutoff dose is likely to be strain dependent and correlates with the dose-response data for the WITS which showed consistent colonization at doses greater than $\sim 10^4$ CFU. The fact that the underrepresented WITS in the biased inoculum experiments can be absent from some birds and groups suggests that the likelihood of a given strain's successfully establishing colonization depends more on the amount of the particular strain in the inoculum rather than the total dose. While our observations can support only the conclusion that founder effects occur in the initial colonization event, similar challenges will face bacteria ingested from the environment during bird-to-bird transmission. Transmission is likely to be mediated by sporadic ingestion of variable numbers of bacteria, and population bottlenecks could contribute to the variability in strain distribution between birds both in this experimental system and during natural spread of *Campylobacter*.

These experiments have led us to consider exactly what is meant by colonization as applied to *Campylobacter*. Clearly, within 24 h postinfection campylobacters have reached the ceca and replicated (total bacterial load is greater than that in the inoculum). Subsequently, it is likely that reinfection from the environment and not solely persistence in the GI tract per se is important for the maintenance of colonization. Phenotypic differences between strains in regard to their ability to persist within groups (29) may not be solely a consequence of their behavior within the GI tract but also their ability to survive outside the host and to transmit to other individuals. Studies seeking to obtain estimates of transmission parameters in the context of models for epidemic spread of *Campylobacter* should begin to explicitly consider the significance of transmission from the environment (5). Additionally, the development of control strategies should consider the possibility of interfering with the transmission process as a method to reduce total flock prevalence.

These observations affect the interpretation and planning of

colonization experiments for *Campylobacter*. For the simplest experiments, for example, where a single mutant is inoculated into groups of chickens and colonization is measured by load in the ceca, then any phenotypic variation or transmission is unlikely to be a confounding factor, although it is important to consider the comments above relating to the nonindependence of birds within a group. If a mutant has a change in its maximal colonization ability, this will clearly be detected. Confounding factors are likely to become significant where there is a mixed population of strains either within individual birds or within a group, for example, where a mutant is competed with a wild type to assess its fitness or during competitive exclusion studies. To minimize the effect of phenotypic variation during mutant construction, multiple experiments should be conducted with independently derived mutants. This is of course a general principle, but data in the literature are often presented from single isolates, presumably due to the requirement to minimize the use of experimental animals.

Perhaps more importantly, the nonindependence of cohoused birds means that events in one individual have the potential to affect the group as a whole. Dose-response studies, in particular, may be affected by this possibility as colonization of only one bird in a group has the potential to rapidly result in the other birds' becoming *Campylobacter* positive. Indeed, it has recently been shown that preventing transmission by performing a dose-response experiment on individually housed chicks significantly modifies the shape of the observed dose-response curve and reduces the uncertainty in estimates of the minimum colonization dose (J. E. Line, and K. L. Hiatt, presented at the 14th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, Rotterdam, The Netherlands; also J. E. Line, K. L. Hiatt, and A. J. K. Conlan, unpublished data). Additionally, cohousing leads to the possibility that bacteria- or host-mediated changes (e.g., phase variation or an increase in shedding) in one individual could result in changes in colonization levels in other individuals within the group. It would therefore be preferable to obtain biological repeats by using larger numbers of groups rather than a larger number of animals within a group, i.e., three groups of three (each group ideally receiving independently derived isolates) rather than a single group of 10. This would minimize the possibility that the results obtained are biased due to in vivo phenotypic changes or transmission within a single cage.

While this study has used a chicken model of colonization, the nonindependence of cohoused animals could be relevant to other models that have been used in competition experiments such as neonatal pigs (25) and MyD88 knockout mice (38), where *Campylobacter* was present in the feces. Additionally, we believe that within- and between-group variations reflect fundamental characteristics of the behavior of *Campylobacter* during colonization and suggest that it would be informative to present data showing the relative loads of different strains within individual animals. Typically, either CIs are calculated and shown as a mean value across a group with a measure of error (4, 25, 34) or the loads of different strains are shown for all individuals in a group without showing the relative proportion within an animal (19, 38).

Finally, it is important to consider that all of the comments above relating to the potential for phenotypic variation and transmission resulting in variable amounts of the competing

WITS also apply to competition between individual organisms within a population of a single strain of *Campylobacter*. The WITS are only distinguishable by virtue of the DNA tags, essentially giving us an insight into the competition occurring between subpopulations within a single strain. The phenotype of a mixture of WITS in vitro and its interaction with the host in vivo are indistinguishable from that of a wild type population. Genetic variability within any population of organisms of course occurs due to random mutation, but the phase-variable tracts of *Campylobacter* have the potential to generate rapidly a number of competing variants. This will result in many competing subpopulations, the relative proportions of which will vary in unpredictable ways. A better understanding of the dynamics of this process should enable improvements in experimental design and the construction of models for the spread and control of *Campylobacter*.

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